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Neprilysin Deficiency-Dependent Impairment of Cognitive Functions in a Mouse Model of Amyloidosis

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Abstract Alzheimer's disease, responsible for the vast majority of dementia cases in the elderly population, is caused by accumulation of toxic levels of amyloid β peptide ($A\beta$) in the brain. Neprilysin is a major enzyme responsible for the degradation of $A\beta$ in vivo. We have previously shown that elevation of neprilysin levels in the brain delays the deposition of $A\beta$ -plaques in a mouse model of amyloidosis and that lack of neprilysin leads to increased $A\beta$ generation and to signs of incipient neurodegeneration in mouse brains. This study was designed to test whether low brain levels of neprilysin affect the amyloid pathology or perturb the learning and memory performance of mice. Double-mutated mice carrying a targeted depletion of one allele of *Mme*, the gene encoding neprilysin, and over-expressing human amyloid precursor protein (APP), exhibited a reinforced amyloid pathology in comparison with their APP transgenic littermates. Moreover, in contrast to their parental lines, these mice were impaired in the Morris water maze learning and memory paradigm and showed facilitated extinction in the

conditioned taste aversion test. These data suggest that even a partial neprilysin deficiency, as is found during aging, exacerbates amyloid pathology and may impair cognitive functions.

Keywords Alzheimer · $A\beta$ · Cognition · Therapy · Amyloid pathology · APP

Introduction

Accumulation of $A\beta$ in the brain is viewed as the primary cause of Alzheimer's disease (AD) pathogenesis. $A\beta$ accumulation is proposed to result from an imbalance between $A\beta$ production and clearance and to induce the formation of neurofibrillary tangles [1]. The hippocampal formation is one of the first areas affected by AD and hippocampal CA1 and CA3 neurons are instrumental for learning and memory in rodents, nonhuman primates, and humans [2–5]. The finding that familial AD is caused by mutations in genes encoding the amyloid precursor protein (APP) and presenilins, leading to high production of the $A\beta$ peptides in the brain, has established the importance of over-production of $A\beta$ in AD pathophysiology. Consequently, mouse models were generated that produce $A\beta$ pathology in an age-dependent manner. In contrast, a higher production rate of $A\beta$ has not been found in sporadic AD, which comprises more than 90% of AD cases. Therefore, the cause of accumulation of $A\beta$ in sporadic cases is likely to be a deceleration of $A\beta$ degradation [6, 7].

Neprilysin, a member of the metalloprotease family, was identified as the major physiological $A\beta$ -degrading enzyme in the brain [8, 9]. Neprilysin levels in AD brain regions with high plaque content have been shown to be approximately half high as the levels found in control brain

Special issue to Honor Dr. Akitane Mori.

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areas with low or moderate plaque burden [10, 11] and furthermore correlated inversely with amyloid load in mouse brains [12]. Mechanistically, the presence of neprilysin at presynaptic sites was shown to be of particular importance in protecting synapses from A β -derived synaptotoxicity [13]. We have recently demonstrated that higher brain neprilysin activity delayed the deposition of amyloid plaques in a mouse model of amyloidosis, whereas, neprilysin deficiency led to A β accumulation and amyloid deposition in mouse brains [14, 15].

The objectives of this study were to determine whether low brain levels of neprilysin affect A β concentrations and amyloid plaque pathology in the brains of transgenic mice over-expressing human A β , and whether altered amyloid pathology reduces the performance of these mice in behavioral tests. We report here that even a reduction of brain neprilysin activity to half of normal levels induced an accelerated accumulation of A β in mouse brains associated with significant impairments in hippocampal-dependent and independent cognitive tasks.

Experimental Procedures

Mice

Both parental mouse lines, with targeted depletion of the neprilysin gene [16] or the J20 mouse line with transgenic neuronal over-expression of human APP (APPtg) [17], were crossbred for more than 20 generations with the C57BL/6 background. Mice were housed under a light cycle of 12 h, with free access to dry food pellets and water, and were weaned at 3 weeks of age. Cross-breeding of the mouse lines was performed by breeding male APPtg mice with female mice heterozygous for neprilysin depletion to generate wild-type (WT), APPtg, mice heterozygous for neprilysin deficiency (NEP $-$) and double-mutated mice heterozygote for neprilysin deficiency and over-expressing human APP (NEP-APPtg). The presence of the neprilysin knock-out versus WT allele and the APP transgene were determined as previously described [14, 17, 18].

First mouse groups were tested in the open field and Morris water maze (MWM) behavioral tests, and then were analyzed by biochemical and immunohistochemical methods. A second group of mice was tested in the conditioned taste aversion (CTA) test. All experiments were performed following national and cantonal regulations and procedures as approved by the cantonal ethics committee.

Behavioral Studies

A total of 48 2 year-old mice ($n = 9$ –14 per genotype), including balanced numbers of male and female subjects,

were subjected to first round of behavioral testing. After assessing activity in an open field arena, mice were subjected to the MWM test, which measures hippocampus-dependent spatial learning and memory. Finally, a second group of mice, originating from the same breeding pairs ($n = 10$ per genotype, containing male and female mice, 24 ± 1 months of age), were subjected to the CTA test.

Morris Water Maze

A modified MWM protocol was employed as described previously [2]. Briefly, training and testing of mice were performed under 12 lux diffuse light in a circular pool constructed from white polypropylene (diameter 150 cm, wall height 50 cm), filled with opaque water to a height of 16 cm and maintained at 24–26°C. In the cued or visible platform learning, mice were trained for 2 days (six trials per day) to find a 16 cm \times 16 cm target platform of white wire mesh, which was placed 0.5 cm above the surface of water and the position of which was changed with each trial. Subsequently, to examine the spatial learning, the platform was submerged 0.5 cm below the water surface in the NW, NE, SE or SW quadrant 35 cm from the pool wall. Each mouse was released to the pool from different starting points and was trained to locate the platform, which was maintained in the same position for an individual mouse, over 4 days with six trials per day separated by 30–60 min intervals and each trial lasting maximally 120 s. About 24 h after completion of training, mice were subjected to a probe trial of 30 s without the goal platform to test spatial retention. Animals were tracked using a Noldus Etho-Vision 3.0 video-tracking system (www.noldus.com) at 576 \times 768 pixels spatial resolution and 4.167 Hz sampling frequency. Raw XY coordinates were transferred to custom-developed public domain software Wintrack [21] (www.dpwolfer.ch/wintrack) for further analysis. Spatial selectivity during the probe trial was assessed using: (1) percentage time spent in a circular zone centered on the trained goal and comprising one-eighth of the pool surface (target zone) versus control zones in the other quadrants, and (2) the number of crossings over the target area and respective control annuli of 16 cm diameter.

Conditioned Taste Aversion Test (CTA)

Water-deprived mice ($n = 10$ per genotype) were housed individually and were trained over four consecutive days to drink from two drinking tubes for 20 min in the morning and 10 min in the afternoon, with a 4 h inter-trial interval [14, 18]. The fluid intake for each mouse was determined by weighing the drinking tubes before and after each trial. On the conditioning day, mice were allowed to drink for 20 min from only one tube, which was filled with a 0.5%

saccharin solution (Fluka, Buchs, Switzerland) (conditioned stimulus). Mice were then administered a 0.14 M LiCl solution (at 0.2% of body weight, ip) as the nausea-inducing agent (unconditioned stimulus) 40 min later. About 48 h after the conditioning trial, animals were subjected to the first preference choice test (retrieval trial), which constituted presentation of two drinking tubes, one containing 0.5% saccharin and one containing water (choice test) for 20 min. The aversion coefficient was calculated as the percentage of saccharin consumption compared with the total fluid intake. This parameter correlates inversely with conditioned learning and memory performance. This test was repeated once per week over four consecutive weeks. Finally, the normal taste sensitivity of mouse groups was determined by testing the natural aversion towards a bitter taste by offering the mice 0.02% quinine solution (Fluka, Buchs, Switzerland) or water [22, 23].

Biochemical Analysis

Following behavioral testing, 7–8 mice per genotype were randomly chosen for biochemical analysis of brain tissue. Mice were perfused transcardially with ice cold PBS, whole brains were removed, followed by removal of the cerebellum and olfactory bulbs. Brains were first cut sagittally and then at bregma level dividing each hemisphere into a frontal region and the caudal brain tissue containing the hippocampus. The frontal parts and one hippocampus-containing sample derived from each brain were frozen immediately. The remaining caudal sample of each brain was fixed for 48 h in 4% paraformaldehyde at 4°C, washed several times in PBS and embedded in paraffin. The presence of amyloid plaques was determined in coronal sections including the hippocampus at interaural level –1.5 mm to bregma, utilizing the 4G8 monoclonal antibody (Serotec) recognizing both human and murine A β as described [19]. The second sample of caudal brain tissue, containing the other hippocampus, was weighed and placed in ice-cold buffer (1:15 w/v) containing 100 mM Tris (pH 8.0), 150 mM NaCl, 2% SDS and complete protease inhibitor cocktail (Roche), then homogenized at 1,000 rpm using a Potter S homogenizer. Brain homogenates were then centrifuged at 100,000g for 1 h at 4°C. The supernatant was collected and the remaining pellet was dissolved in 70% formic acid (1:3 w/v). The concentrations of A β 40 and A β 42 were measured in the brain homogenates using commercially available kits measuring both human and murine A β species (Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to the manufacturer's instructions.

In addition, neprilysin enzymatic activity was measured in the frontal brain tissues, following homogenization in a buffer containing 100 mM Tris (pH 8.0) and 150 mM

NaCl, according to published methods [19, 20]. Background absorbance readings were established using brain homogenates ($n = 4$) prepared from age-matched neprilysin knock-out mice [16].

Statistical Analysis

Data were collected by colleagues unaware of the genotypes of the mice. For behavioral tests, groups were always trained and tested in parallel and identically. Statistical analysis of biochemical and CTA data was performed using one way analysis of variance (ANOVA) and significant main effects of genotype were further analyzed using Fisher's PLSD post-hoc test. For analysis of MWM performance, the data of all groups including males and females, were analyzed using a 3-way factorial ANOVA model with the between-subject factors of APPtg+ (APPtg vs. WT), neprilysin heterozygosity (NEP– vs. WT) and sex. No gender-dependent effects were found. Therefore, final analysis was performed using a two-way ANOVA without the gender factor. In order to fit the factorial ANOVA model, training variables were averaged over all training trials. For selected variables, the ANOVA model was complemented by the within-subject factors of time (blocks of two subsequent trials) and place (trained vs. average of non-trained sites), in order to assess learning rates and place preferences. To further explore potential interactions between the mutations, the ANOVA model was split along the APPtg+ or NEP– factor. Data are expressed as mean \pm SEM, ns shows a non-significant effect.

Results

Neprilysin Deficiency was Associated with Increased Amyloid Pathology

Amyloid plaque load was studied in mouse brains via immunohistochemistry utilizing the 4G8 antibody, which recognizes both human and mouse A β peptides. APPtg mice exhibited small punctated and compacted amyloid plaques, whereas, the number and size of amyloid plaques were greater in the brains of doubly mutated NEP-APPtg mice (Fig. 1). Concentrations of SDS- and formic acid (FA)-soluble A β 40 and A β 42 were further quantified in each mouse brain (Table 1). In SDS homogenates of WT brains, the A β 40 concentration was 0.69 ± 0.05 nM, whereas, A β 42 values did not exceed background levels. A β 40 concentrations differed markedly between groups in the following manner: WT < NEP– < APPtg < NEP-APPtg (Table 1). In the FA homogenates, however, measurable A β 40 was only found in APPtg and NEP-APPtg brains with levels in the latter genotype being

Fig. 1 Neprilysin deficiency exacerbates amyloid pathology in mouse brains. Frontal brain sections through the hippocampal formation were stained with the 4G8 antibody, which recognizes both human and murine A β . APPtg mice exhibited distinct amyloid deposits but the amyloid plaque load was highest in doubly-mutated mice (NEP-APPtg) combining chronic low neprilysin levels and expression of the AD-associated human APP transgene

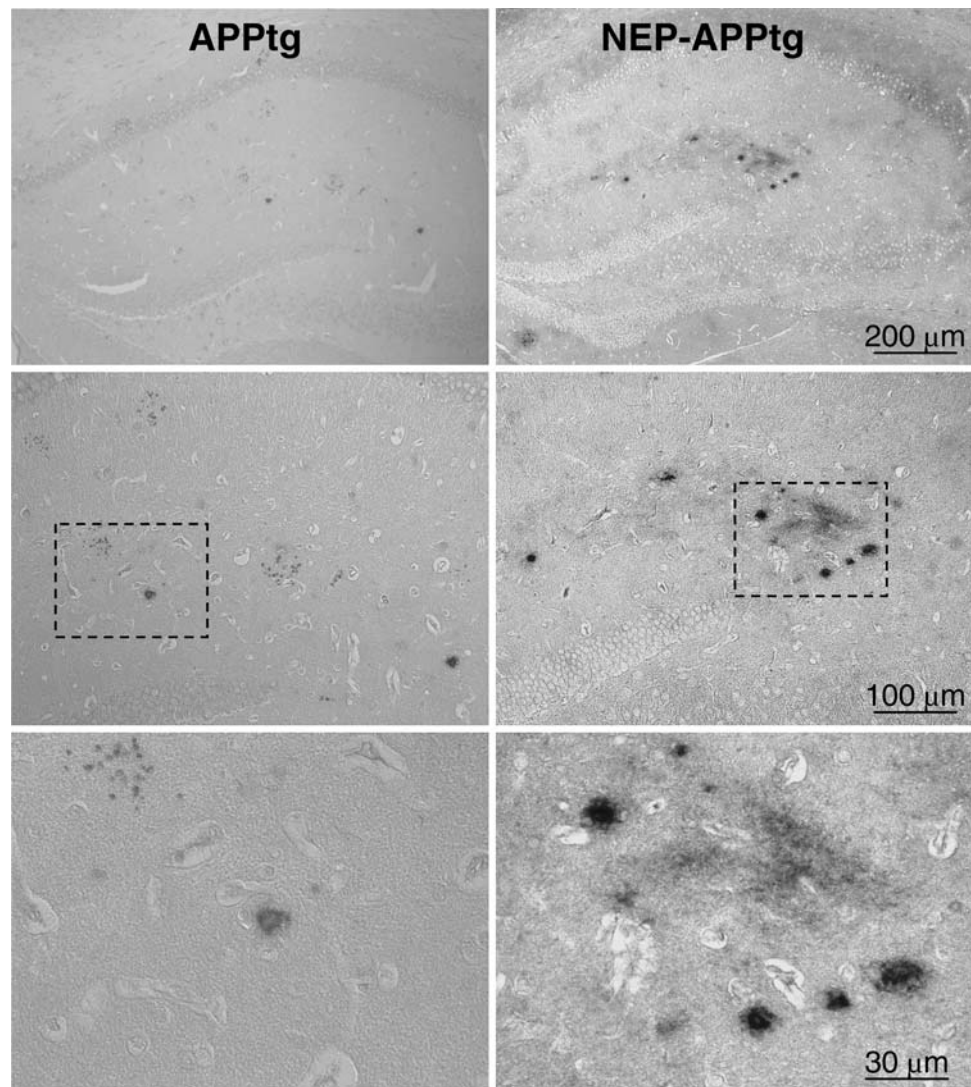


Table 1 Quantification of brain A β concentrations

	WT	NEP–	APPtg	NEP-APPtg
SDS soluble				
A β 40 (nM \pm SEM)	0.69 \pm 0.05	1.27 \pm 0.09	2.07 \pm 0.1	5.79 \pm 0.25
A β 42 (pM \pm SEM)	Nd	Nd	Nd	1,067 \pm 161
Formic acid soluble				
A β 40 (nM \pm SEM)	Nd	Nd	0.383 \pm 0.026	1.049 \pm 0.10
A β 42 (pM \pm SEM)	Nd	Nd	Nd	887 \pm 290

Brain A β levels (both murine- and transgenically expressed human-species) showed that lowered neprilysin levels or expression of AD-causing human APP led to an accumulation of A β in mouse brains

Levels of A β 40 and A β 42 were below detection sensitivity in the formic acid fraction of WT and neprilysin-deficient mice. Moreover, A β 42 was not detected in brains of WT mice, confirming previous findings that these levels are too low for detection by ELISA [51, 52]. Finally, the entire A β content in brains of the APPtg mice represented A β 40, reflecting the mild amyloid pathology in our APPtg mouse line, when compared to their NEP-APPtg littermates. The concentrations of SDS- and formic acid-soluble A β 40 in brains of NEP-APPtg mice were significantly higher than the corresponding levels in the APPtg littermates ($P < 0.01$ and < 0.001 , respectively)

Nd not detectable

approximately 2.7 fold higher than in the APPtg brains. Furthermore, no FA-soluble A β 42 was found in WT, NEP– and APPtg brains (Table 1). As expected, neprilysin enzymatic activity was reduced in NEP– and NEP-APPtg mice when compared to their WT or APPtg littermates (Fig. 2).

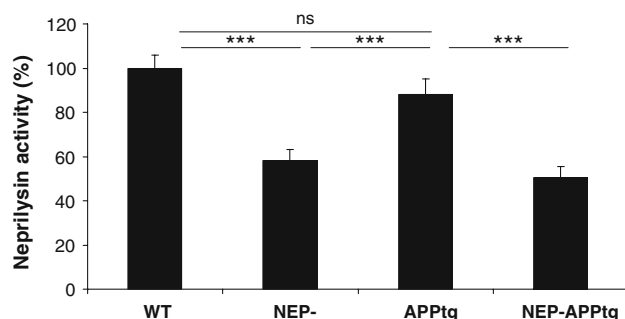
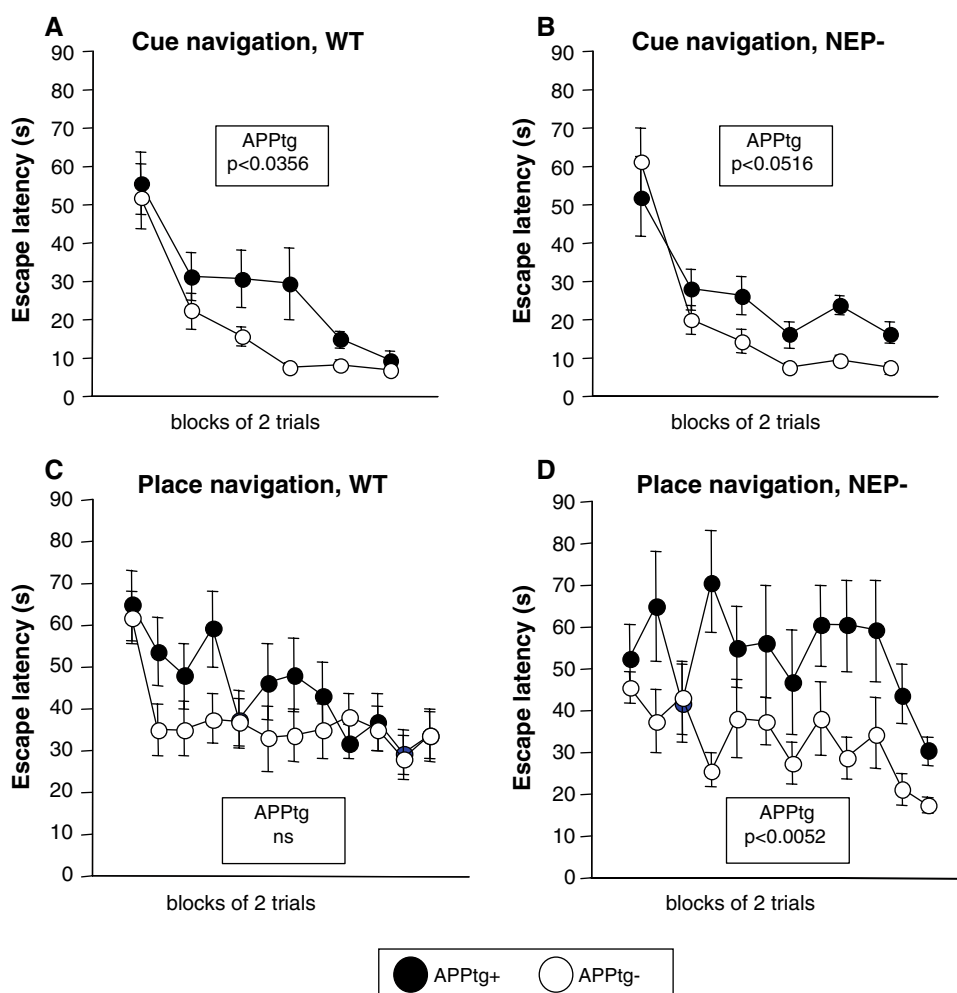


Fig. 2 Neprilysin enzymatic activity was significantly lower in NEP– and NEP-APPtg brains when compared to their WT or APPtg littermates (enzyme activity in WT brains was set at 100%). No statistical difference was observed between the WT and APPtg groups. *ns* not significant, *** $P < 0.0001$

Fig. 3 APP transgene effect without (a, c) and with (b, d) concomitant neprilysin deficiency on training performance in the Morris water maze. **a, b** All groups learned the cue navigation task. The escape latencies (in seconds) of each two consecutive trials were calculated and the group averages were built. Neprilysin deficiency did not affect the performance in the cue navigation task, but independently of the neprilysin expression level the inheritance of APP transgene was associated with a small increase in escape latencies. (for significance levels see the “Results” section). **c, d** In the place navigation task, the data are suggestive of a NEP deficiency dependent effect of the APP transgene on escape performance. The escape latencies are built as above. The APP transgene was associated with significantly increased escape latencies in neprilysin deficient mice (d). In the presence of normal neprilysin levels (c) this difference was much smaller and statistically insignificant (for significance levels see the “Results” section)



In addition, no statistical difference was found in the WT and APPtg brains suggesting that over-expression of human APP did not affect brain neprilysin activity (1-way ANOVA: genotype $P < 0.0001$, WT vs. NEP– $P < 0.0001$, WT vs. APPtg *ns*, WT vs. NEP-APPtg $P < 0.0001$, APPtg vs. NEP-APPtg $P < 0.0001$, Fig. 2).

Neprilysin Deficiency Resulted in Worsening of Hippocampus-Dependent Cognitive Functions

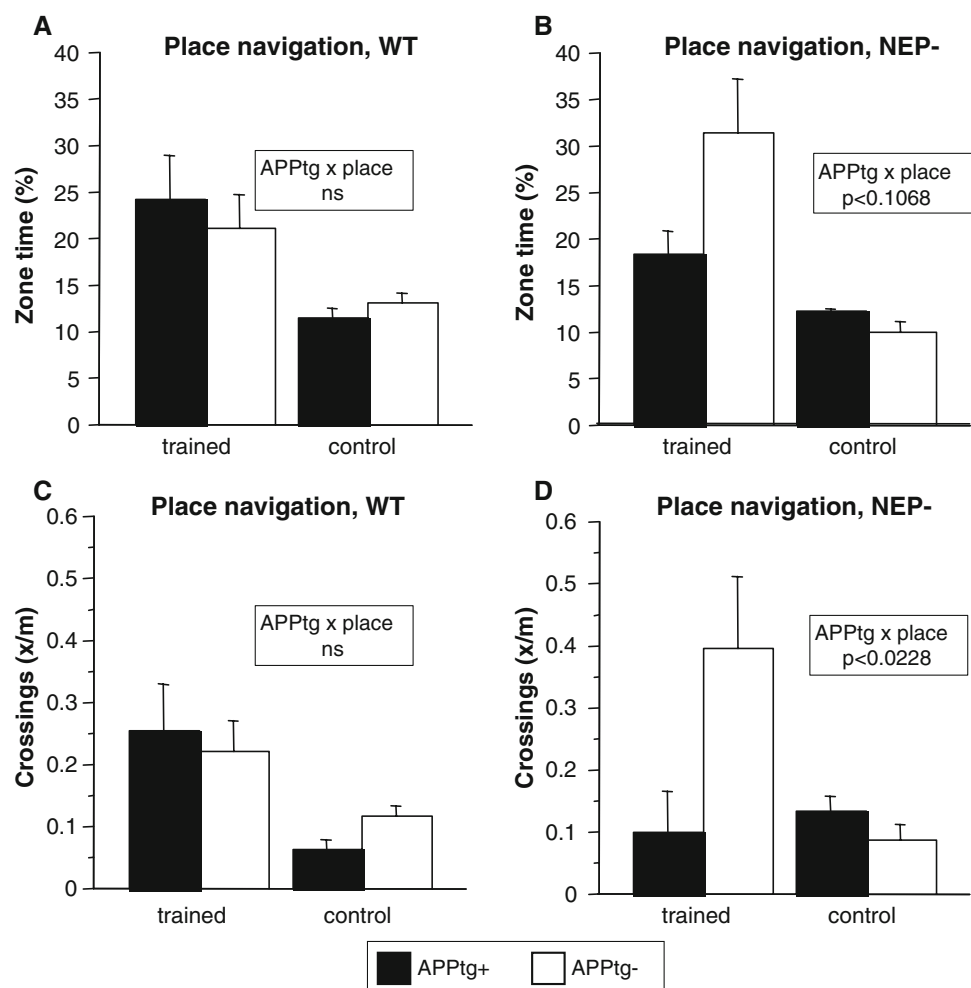
In the cued navigation task, all groups learned to find the visible platform. In this task, independent of the NEP expression level, the APP transgene was associated with a small increase in escape latencies, while neprilysin deficiency itself had no effect (repeated ANOVA: APPtg+ $P < 0.0055$, NEP– *ns*, time $P < 0.0001$, APPtg+ \times time *ns*, NEP– \times time *ns*, NEP-APPtg *ns*, Fig. 3a, b). In contrast, in the place navigation task, we found that the effect of the APP transgene on escape performance depended on neprilysin deficiency (Fig. 3c, d). The presence of the APP transgene was associated with significantly

increased escape latencies in NEP[−] mice (Fig. 3d), while in the presence of normal neprilysin levels this difference was statistically insignificant (Fig. 3c) (repeated ANOVA: AP-Ptg⁺ $P < 0.0023$, NEP[−] ns, time $P < 0.0001$, AP-Ptg⁺ \times time ns, NEP[−] \times time $P < 0.0749$, NEP-AP-Ptg ns; split ANOVA AP-Ptg⁺: in WT place ns; in NEP[−] $P < 0.0052$, Fig. 3c, d).

The genotype affected spatial measures directly related to memory and cognition, as assessed in the probe trial (Fig. 4). Performance during the probe trial, undertaken following the MWM place navigation task, showed that all mice searched preferentially in the target zone, thus indicating successful spatial learning. Time spent in the target zone further indicated an effect of neprilysin-deficiency on spatial selectivity that was dependent on the APP transgene. The NEP-AP-Ptg mice exhibited a reduced selectivity for the target zone when compared with their AP-Ptg littermates (repeated ANOVA: place $P < 0.0001$, AP-Ptg⁺ \times place ns, NEP[−] \times place ns, NEP-AP-Ptg \times place $P < 0.0790$; split ANOVA AP-Ptg⁺: in WT place $P < 0.0045$, AP-Ptg⁺ \times

place ns; in NEP[−] place $P < 0.0057$, AP-Ptg⁺ \times place $P < 0.1068$, Fig. 4a, b). Moreover, analysis of annulus crossings, the most stringent measure of spatial selectivity, confirmed that the APP transgene was associated with a loss of spatial selectivity in the presence (Fig. 4d), but not absence of (Fig. 4c), neprilysin deficiency (repeated ANOVA: place $P < 0.0031$, AP-Ptg⁺ \times place $P < 0.1076$, NEP[−] \times place ns, NEP-AP-Ptg \times place $P < 0.0228$; split ANOVA AP-Ptg⁺: in WT place $P < 0.0080$, AP-Ptg⁺ \times place ns; in NEP[−] place $P < 0.1032$, AP-Ptg⁺ \times place $P < 0.0228$, Fig. 4c, d). AP-Ptg mice were unimpaired in MWM testing, a result that can be explained with the mild A β pathology in our mouse line and in agreement to previous results [18]. Fig. 4 seems to suggest such an improvement in the NEP[−] animals. However, this trend was statistically not significant (comparison of NEP^{+/+} vs. NEP[−] in the AP-Ptg[−]: place $P < 0.0079$, place \times NEP[−] $P < 0.1509$). Probe trial measures were normalized to distance swum to avoid interference with differences in swim speed.

Fig. 4 APP transgene effect without (a, c) and with (b, d) concomitant neprilysin deficiency on performances during the probe trial. **a, b** The spatial selectivity was assessed by the percent of time spent in the target zone area. The APP transgene was associated with reduced selectivity in the presence (b) but not in the absence (a) of neprilysin deficiency suggesting a neprilysin-deficiency dependent APP effect (for significance levels see the “Results” section). **c, d** According to assessment of annulus crossings, the most stringent measure of spatial selectivity, defined by the number of crossings over the target area and respective control annuli per swam distance, the APP transgene was associated with a loss of spatial selectivity in the presence (d) but not in the absence (c) of neprilysin deficiency (for significance levels see the “Results” section)



Neprilysin Deficiency Resulted in Worsening of CTA Performance

Testing mice in the CTA required subjecting mice to water-deprivation. Therefore, to avoid any possible behavioral influences of previous training in the MWM and in order to be able to test the mice in all behavioral tests at ± 24 months of age, we decided to use a separate mouse group for the CTA rather than using the same mice that were tested in the MWM. Age-matched mice ($n = 10$ each genotype), originating from the same parents as mice tested in the previous behavioral tests were employed to examine the hippocampus-independent associative memory in CTA paradigm.

Body weight of the mice did not vary significantly between groups during the course of the CTA test. Moreover, all groups drank comparable amounts of water during the adaptation- and conditioning-phases and genotype did not affect the amount of saccharin drunk during the conditioning trial. 48 h after conditioning, mice were subjected to the first choice test (Fig. 5, day 1), by presenting them with one bottle containing saccharin solution and one bottle filled with water. No differences in memory trace were found between the genotype groups in the first choice test,

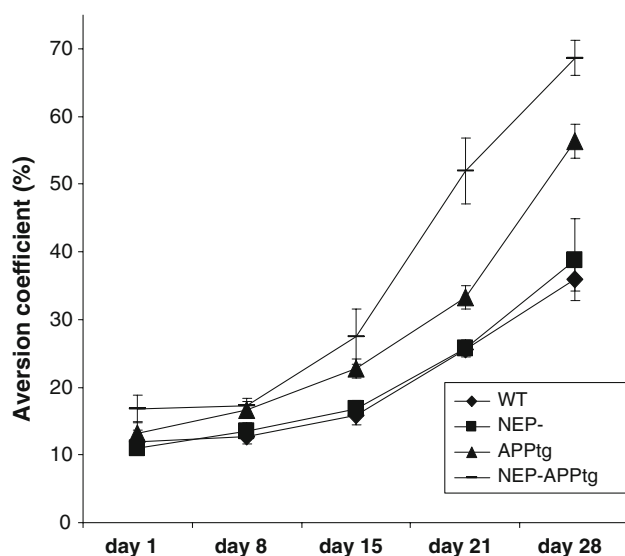


Fig. 5 Effects of APP transgene and neprilysin deficiency on memory performance in the CTA. The aversion coefficient, a measure of impairment of associative memory, defined as the percentage of saccharin consumption divided by the total fluid intake, is depicted over five choice tests. All genotype groups succeeded in CTA but saccharin intake was significantly increased in the presence of the APP transgene. Both APPtg and NEP-APPtg mice displayed a significantly diminished conditioned aversion against saccharin compared to NEP- and WT mice, with NEP-APPtg mice exhibiting the poorest memory performance. Performance of NEP mice did not differ from their WT littermates (for significance levels see the “Results” section)

indicating that all groups learned the task successfully (Fig. 5, day 1). The strength of the conditioned aversion ($P < 0.001$) and the speed of its extinction were significantly different (genotype \times days $P < 0.001$) among the genotype groups (Fig. 5). The APP transgene was associated with facilitated extinction of the associative memory. During the subsequent weekly choice tests (Fig. 5, days 8, 15, 21, 28) an increase of aversion coefficient, i.e., memory impairment, was observed in all groups. WT and NEP-mice displayed an equally strong aversion against saccharin, suggesting that neprilysin deficiency did not alter the CTA performance. APPtg as well as NEP-APPtg doubly mutated mice showed a significantly weaker conditioned aversion in the choice tests with NEP-APPtg showing the lowest CTA performance (ANOVA: genotype $P < 0.001$, WT vs. NEP- ns, WT vs. APPtg $P < 0.001$, WT vs. NEP-APPtg $P < 0.001$, APPtg vs. NEP-APPtg $P < 0.01$). Taken together, these results show a negative effect of the APPtg genotype on CTA performance, which is further worsened by neprilysin deficiency.

Lastly, the avoidance of an intrinsic aversive bitter taste was determined by offering the water-deprived mice quinine solution or water. No difference between the four groups was detected ($P < 0.48$, not shown), confirming that taste sensitivity did not differ between the groups.

Discussion

The findings described in this paper show that partial lack of neprilysin, resembling the lowered neprilysin levels in aged human brains, led to an enforced amyloid pathology in the brain and to impairment of both hippocampal-dependent and-independent cognitive performance.

Accumulation of $A\beta$ in the brain is associated with memory impairment of AD patients. High $A\beta$ levels are shown to induce synaptic dysfunction [1, 17, 24] and to result in the formation of amyloid plaques, leading to compromised neuronal integrity and function [25–27]. The topological and spatial distribution of neprilysin suggests a physiological role in $A\beta$ catabolism [7, 28]. In addition, neprilysin is down-regulated in the hippocampus and cerebral cortex during normal aging [29, 30] and in early stages of AD development [10, 29, 31], thus linking an inefficient $A\beta$ clearance from the brain and AD pathophysiology. Moreover, neprilysin has been shown to be a major peptidase capable of $A\beta$ degradation in vivo [8, 9].

An important contribution to understanding the mechanisms of AD pathophysiology was achieved by generation of transgenic models over-expressing APP in brain neurons. Despite the importance of these mice in mimicking amyloid plaque pathology associated with AD, it should be kept in mind that not only $A\beta$, but also APP and APP-proteolytic

fragments are over-expressed and thus accumulate in the brains of such models, making it difficult to assign the observed effects to $A\beta$ only. This difficulty arises from the fact that APP proteolytic fragments are implicated in contradictory functions: they can be neurotoxic [32–34], may impair LTP [35, 36], lead to neurodegeneration [37, 38] and cognitive impairments [36, 37], while also being reported to enhance LTP [39], to be neuroprotective [40] and to up-regulate neprilysin activity [41].

A further complication arises from the fact that deviations from physiological APP level may have negative functional consequences. It was shown, in mice over-expressing APP, that $A\beta$ rather than APP is involved in synaptotoxicity as measured by assessment of synaptophysin immunoreactivity [17]. Counter-intuitively, a similar decrease in synaptophysin immunoreactivity, impaired synaptic plasticity and deficits in cognitive functions were also found in mice lacking APP [42–44] and a defective memory performance was also reported in mice over-expressing WT APP [45]. These data demonstrate the complex role of APP and its photolytic fragments in neuronal function.

Reports from several laboratories, including ours, show that neprilysin degrades $A\beta$ without affecting APP metabolism [13, 14, 18, 46, 47]. Therefore, in the present study, by using mice with lower neprilysin activity we were able to specifically target $A\beta$ without modification of APP processing because APP processing is not changed by neprilysin deficiency [14] or over-expression [18, 46, 47]. Therefore, APP transgenic mice that also carried one defective *Mme* allele represent a unique experimental model to analyze effects mediated by a sole elevation of $A\beta$ on cognition. The reason for employing mice with only a partial neprilysin deficiency, rather than mice with a total lack of neprilysin, was that we aimed to recapitulate the human situation as accurately as possible, in which a reduction—but not elimination—of neprilysin is detected in aged or AD brains. In the double-mutated mice (NEP-APPtg), the neprilysin activity was reduced to approximately half of corresponding values in APPtg brains, reflecting the reduction of neprilysin in brains of AD patients [48, 49]. Moreover, we employed an APPtg mouse line exhibiting a relatively mild phenotype, again to be able to model the accumulation of $A\beta$ in brains of AD patients over an extended period of time.

Biochemical analysis revealed that in comparison with APPtg littermates, the double-mutated mice exhibited a significant decrease in neprilysin enzymatic activity and an elevation of brain $A\beta$. To examine whether neprilysin deficiency affects cognitive functions, mice were tested in MWM and CTA tests, exemplifying hippocampus-dependent spatial learning and memory and hippocampus-independent associative memory, respectively.

In the open field (not shown) we found that motor and motivational characteristics were indistinguishable from each other. This finding is in concert with previous data that APPtg and neprilysin-deficient mice are normal and that the reduction of neprilysin enzymatic activity can be well tolerated by the organism [6, 16] and with own data that neprilysin heterozygous and homozygous mice were very similar in behavioral tests [14].

In all cognitive behavioral tests, neprilysin-deficient mice did not differ from WT controls. In the MWM test, all groups of mice were able to find the location of both a visible and a hidden platform. Moreover, NEP— mice performed similarly to their WT littermates in the probe trial, reconfirming their normal spatial memory and learning performance. In contrast, the combination of neprilysin deficiency with APP over-expression in NEP-APPtg mice was associated with behavioral impairment in the probe trial, a finding that is in line with overt accumulation of soluble and aggregated forms of $A\beta$ in brains of the double-mutated animals. Therefore, inefficiency of $A\beta$ removal from the brain due to a reduced neprilysin activity led to the diminished performance in the hippocampus-dependent MWM.

Our data are in line with reports showing that elevated brain $A\beta$ levels in mice with neuronal expression of mutated human APP or in neprilysin knock-out mice cause a worsening of CTA [14, 22]. Combining neprilysin deficiency and elevated APP expression in NEP-APPtg mice proved most destructive for CTA performance. In agreement with the MWM data, performances of WT and NEP— mice in the CTA test were indistinguishable. Moreover, a negative dominant effect of the APPtg genotype on CTA performance was observed as reported earlier [18], which was accentuated in the NEP-APPtg double-mutated mice. As outlined for the MWM, these results indicate that neprilysin deficiency and the consequent accumulation of $A\beta$ resulted in the inability of the double-mutated mice to perform normally in the CTA testing paradigm.

Recent data suggest that the levels of neprilysin substrates, except for $A\beta$, are unchanged in brains of neprilysin knock-out mice [28], excluding the possibility that observed effects in the NEP-APPtg mice may be due to accumulation of those other neprilysin substrates. Moreover, $A\beta$ degradation by neprilysin is a down-stream event after $A\beta$ generation and does not affect APP metabolism [13, 14, 18, 46, 47, 50]. These data, in combination with the fact that NEP— mice did not differ from WT littermates in their performances in the MWM and CTA behavioral tests, stress the specificity of the observed effects. They also show that a worsening of memory performance of the NEP-APPtg mice in these cognitive paradigms was caused by accumulation of $A\beta$ in the brains of double-mutated mice.

We showed here that a partial reduction of neprilysin activity and the consequent defective removal of A β from the brain led to exacerbation of amyloid pathology resulting in significant deficits of the NEP-APPtg mice in both associative and reference memory tasks when compared to their NEP- and APPtg littermates. These data are in agreement with a report showing that a complete lack of neprilysin at the site of synapses led to impaired synaptic plasticity and behavioral performance [13], and are in line with the reported selective reduction of neprilysin in sporadic AD, particularly in brain areas with a high plaque burden [10, 11], and in normal aging [29].

Taken together, these data provide the unequivocal evidence that even a partial neprilysin insufficiency, similar to the observed reduction of neprilysin activity in aging brains, leads to an aggravation of amyloid pathology and to cognitive impairments in vivo and furthermore emphasize the importance of developing strategies to maintain physiological levels of neprilysin activity during aging and in AD therapy.

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